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TITLE: Evaluation of Altered Stromal/Epithelial Tissue Arrangement of the c-Kit Messaging System in the Control of Breast Cancer

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14. Abstract

Previous studies have shown that there is a progressive loss of c-Kit expression in breast epithelial tissue as this tissue changes from normal to benign to cancerous, while simultaneously there is a gain in c-Kit expression in the surrounding stromal tissue. We have studied this inversion in c-Kit expression in an organotypic environment in vivo and in culture to determine its influence on tumor growth and tumor chemosensitivity. Human breast cancer cells (Hs578T) and autologous surrounding fibroblasts (Hs578Bst) were admixed and grown as xenografts in the mammary fat pad of immune deficient mice and in culture on a layer of collagen I in Mat Tek dishes. Overexpression of c-Kit in fibroblasts was carried out using Lipofectamine-mediated transfection of pcDNA3/c-Kit. Chemosensitivity to the c-Kit modulating drugs, imatinib and alpha-fetoprotein-derived peptide (AFPeP), was evaluated. Xenografted tumor cells grew only when injected in admixture with fibroblasts. Growth was accentuated when fibroblasts overexpressed c-Kit. Both imatinib and AFPeP inhibited tumor growth when tumor was inoculated with its normal fibroblasts. However, within the context of c-Kit-overexpressing fibroblasts, tumor lost sensitivity to growth inhibition by imatinib but not to AFPeP. In culture, tumor became more rounded when grown in the context of fibroblasts. In the presence of imatinib tumor growth and migration was inhibited and fibroblasts were induced into a dendritic morphology. Overexpression of c-Kit in fibroblasts prevented the imatinib-induced changes in morphology and migration. In the presence of AFPeP, tumor growth was inhibited, migration was partially inhibited, but there were no changes in tumor or fibroblast morphology; and overexpression of c-Kit in fibroblasts did not significantly impact response of tumor to AFPeP. The results of this study indicate that the presence of stroma and particularly c-Kit expression in stroma can influence the growth rate of breast cancer as well as the response of that cancer to targeted therapy.

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Introduction

The purpose of this research is to evaluate stromal influence on breast cancer growth, particularly the role of c-Kit expression in the stroma on the growth and migration of breast cancer cells. There are several reasons to focus on the stroma in the study of breast cancer development, growth and progression. Studies in rats have shown that non-malignant stroma is needed for progression of mammary epithelium from normalcy to malignancy [1,2]. As human breast cancers develop, there is a gain of c-Kit expression in the surrounding stroma, and there is less c-Kit expression in breast cancer epithelia compared to normal breast epithelia [3,4]. Moreover, it has been found that overexpression of stromal c-Kit can sensitize the breast epithelial cells to other growth factors such as epidermal growth factor [5]. Therefore we studied this relationship of breast cancer to stroma, particularly c-Kit expression/function in stroma, by enhancing/inhibiting c-Kit in stroma, admixing these cells in vivo and in culture, and then evaluating the stromal-induced changes in breast cancer growth, morphology, and migration.

Body

We evaluated Hs578T human breast cancer cells with the autologous Hs578Bst breast fibroblasts. This is one of the few human breast cancer/breast stroma pairs from the same patient in which both the breast cancer cells and fibroblasts have been established in culture with both cell lines being commercially available. In vivo, equal numbers of breast fibroblasts and breast cancer cells were admixed and injected into the mammary fat pads of immune deficient (SCID) mice. Time to tumor outgrowth and tumor growth rate were measured. In culture, breast cancer cells and fibroblasts were studied in isolation and in admixture in organotypic environments measuring growth, morphology, and migration of cells.

Hs578T human breast cancer cells have been reported to be non-tumorigenic in immune deficient mice (ATCC web site). We felt we could overcome that obstacle with strategies typically used in our laboratory to grow freshly resected human tumors. We validated that these cells did not grow when injected either subcutaneously or into the mammary fat pad (mfp) of severe combined immune deficient (SCID) or athymic nude mice. Increasing the number of cells in the inoculum from 10^6 to 10^7 did not change this outcome. We solidified these cells in a fibrin clot and transplanted them into the mfp or under the kidney capsule of SCID mice and found no significant tumor growth. We admixed these cells with matrigel, transplanted them into the mfp of SCID mice and obtained within 14 days nodules that measured 1mm to 2mm in diameter but these nodules did not progress in tumor growth. Interestingly, when we admixed Hs578T tumor with Hs578Bst fibroblasts in matrigel, tumor take and tumor outgrowth occurred in 100% of the mice within 7 days and progressive tumor growth occurred thereafter (Fig. 1), clearly demonstrating that the presence of fibroblasts promoted both tumor take and tumor growth. This tumor does constitutively express c-Kit (Densitometry 24317) as does its autologous fibroblasts Densitometry 1375 (Fig. 2). Increased expression of c-Kit in these fibroblasts was accomplished using Lipofectamine-mediated transfection of pcDNA3/c-Kit (Fig. 2). The efficiency of this transfection was modest, increasing the densitometry of the c-Kit band from 13675 to 20838. AKT, a downstream target of c-Kit was also increased modestly (50%) in the transfected fibroblasts (Fig. 3). As shown in Fig. 1A, pharmacological inhibitors of c-Kit inhibited tumor growth. The growth inhibition was significant with AFPep (400 μ g/kg/day i.p.) ($p < 0.05$) but not significant with imatinib (80mg/kg/day i.p.) ($p = 0.1$). AFPep also increased tumor latency by 3 days. The dose and schedule of these agents have been previously reported to be pharmacodynamically effective [6,7]. Interestingly, when these tumor cells were admixed with autologous fibroblasts which had been transfected with c-Kit, the tumor growth rate was

significantly increased (Fig. 1B). AFPep still inhibited the growth and prolonged the latency of this tumor. However, treatment of tumor-bearing mice with imatinib further enhanced the growth rate of this tumor (Fig. 1B).

These are striking results suggesting that autologous fibroblasts can increase tumorigenicity and that overexpression of c-Kit in the stroma can lead to enhancement in tumor growth. However, pharmacologically targeting c-Kit can lead to either tumor growth inhibition or tumor growth enhancement depending on the drug selection and the stromal context surrounding the tumor.

In an effort to explain these results, we evaluated by confocal microscopy Hs578T (tumor) in co-culture with Hs578Bst (autologous fibroblasts). Hs578T was labeled with a red fluorescent dye (CellTracker™ Red CMTPX, Molecular Probes) and Hs578Bst was labeled with a green fluorescent dye (CellTracker™ Green BODIPY®, Molecular Probes). The fibroblasts were suspended in collagen type I and layered onto Mat Tek dishes. The collagen layer was approximately 100µm in thickness. Maintaining a uniform and flat collagen layer was a technical problem that took some time to work out. This was important for achieving uniform depth measurements in this model. The fibroblasts grown in this collagen layer were elongated and spread out (Fig. 4A). This morphology changed in c-Kit transfected fibroblasts in which the cells appeared more rounded (Fig. 4B). Vector-transfected fibroblasts were elongated and spread out. When tumor cells were placed on top of the collagen layer devoid of fibroblasts, the tumors were also elongated and spread out with projections that formed networks between the cells (Fig. 4C). Interestingly, when tumor cells were placed on a fibroblast-containing collagen layer, both tumor and fibroblasts were rounded with no projections or networks between cells (Fig. 5A). This contextual cytoarchitectural change in morphology also occurred with tumor mixed with c-Kit transfected fibroblasts (Fig. 5B). The rounding up of both cell types occurred within the first 24 hours after their co-culture. This morphology maintained itself at later time points (48-72 hours) during which time there was clear evidence of tumor migration down into the fibroblast layer (Fig. 5C,D). This rounded morphology is suggestive that the cells are more proliferative when they are admixed. Flow cytometry data also suggested that the cells are more proliferative when they are admixed. However, in the flow cytometry experiments, the cells lost their stain rather quickly over time, so the proliferative rates were rather difficult to assess. Nevertheless, increased roundedness, increased c-Kit, and increased proliferative activity are all consistent with the increased tumorigenicity and increased tumor growth rate when tumor cells were transplanted in admixture with their associated fibroblasts. Addition of AFPep to this co-culture model did not seem to alter the morphology or the migration pattern of the tumor and fibroblasts (Fig. 5E,F). However, upon addition of imatinib to the co-culture model, cells reverted to an elongated/dendritic pattern and tumor was inhibited from migration into the fibroblast layer (Fig. 5G). Interestingly, if the fibroblast layer was overexpressing c-Kit, the imatinib effect was muted (Fig. 5H).

We explored further this imatinib effect on migration in a Boyden chamber-type migration assay. Cells were seeded on top of a Matrigel-coated membrane. Fibroblasts did not migrate through this membrane whereas tumor did migrate through the membrane in response to serum (5%) being placed into the medium of the lower chamber (Fig. 6A). Fibroblasts did not impede the migration of tumor. Both imatinib and AFPep inhibited migration in a dose dependent manner (Fig. 6B,C); so the results in this assay were partly consistent with the confocal assay in which tumor was seeded on top of a layer of collagen I embedded with fibroblasts. The inhibitory effect of these drugs on migration was not due to a cytotoxic effect of these drugs as AFPep was growth inhibitory only to Hs578Bst and was not cytotoxic even at 10^{-4} M to Hs578Bst, c-Kit Hs578Bst or Hs578T. Imatinib was growth inhibitory to Hs578Bst, c-Kit

Hs578Bst, and Hs578T at concentrations up to 10^{-5} M but did not cause a lytic response in the cells until the concentration was escalated to 10^{-4} M (Fig. 7A-C). Significant inhibition of migration occurred at concentrations (10^{-6} M) well below this cytotoxic level.

Limitations and Alternative Approaches

One of the unanticipated limitations of this investigation was the finding that the Hs578Bst fibroblasts had a slow doubling time (96hr – 120hr), and they began to senesce within 6 generations after we received them from ATCC. This made it difficult to build these cells up to large cell numbers for experiments and impractical to permanently infect them with genes for c-Kit and genes for fluorescent proteins, and also to use siRNA mediated knockdown of c-Kit using retroviral transduction systems since optimal retroviral expression requires active proliferation. This could be remedied in future studies by immortalizing the fibroblasts using SV40 large T antigen system. Of course the trade-off here would be further altering autologous tissue obtained from a breast cancer patient. Nevertheless, if these cells were immortalized, one could then stably transfect them with c-Kit using the tetracycline inducible Lentivirus infection system which would allow one to dissect at will on a timeline the effect of upregulating c-Kit in fibroblasts by simply administering tetracycline. These studies were beyond the scope of the budget and timeline of this concept award but certainly should be considered when designing future studies investigating the influence of stromal c-Kit on breast cancer growth. Nevertheless, we do feel that we accomplished most of the goals of this study and certainly obtained significant high impact results using the transient transfection and staining systems in shorter time duration experiments described herein.

Key Research Accomplishments

1. The tumorigenicity of Hs578T human breast cancer cells in SCID mice is significantly enhanced when it is transplanted in the presence of its autologous (Hs578Bst) fibroblasts.
2. Increase of c-Kit expression in fibroblasts surrounding autologous tumor enhances in vivo growth rate of tumor.
3. Both imatinib and AFPep (known pharmacological inhibitors of c-Kit) inhibited tumor growth when tumor was inoculated with its normal fibroblasts. Tumor chemosensitivity to imatinib but not to AFPep was lost when c-Kit was increased in the surrounding fibroblasts.
4. Co-culture of tumor with fibroblasts resulted in a change in tumor morphology to a more rounded appearance suggesting a more proliferative profile.
5. Imatinib and AFPep inhibited tumor invasion. The anti-invasive property of imatinib was lost with increased c-Kit expression in the co-cultured fibroblasts.
6. Chemosensitivity of breast cancer to the growth inhibitory effects of imatinib was lost when the surrounding stroma overexpressed c-kit.

Reportable Outcomes

1. Abstract submission and publication in the Proceedings of the Era of Hope Department of Defense Breast Cancer Research Program Meeting. June 2008. Baltimore, MD. Abstract # P69-7. p. 465 of Proceedings.
2. Poster presentation to meetings participants and members of the Poster Tour at the Era of Hope Department of Defense Breast Cancer Research Program Meeting, June 27, 2008, Baltimore, MD.

Conclusion

Stroma surrounding a breast cancer, particularly overexpression of c-Kit in that stroma, can enhance the progression of that breast cancer. Targeting c-Kit with drugs that inhibit the function of this tyrosine kinase growth factor receptor may or may not lead to a therapeutic outcome *in vivo* depending on the level of c-Kit expression in the stroma, even though these drugs may be found to inhibit breast cancer cell growth in culture. Studying the effect of imatinib on breast cancer growth in cell culture suggested that this agent may be effective for the treatment of this cancer in patients. However, when the stroma (especially stroma overexpressing c-Kit) was factored into models of breast cancer growth, the sensitivity of breast cancer to imatinib was altered. This change in chemosensitivity also occurred in *in vivo* models of breast cancer growth. The tumor/stroma relationship and growth factor cross talk between these tissues needs to be studied further in organotypic environments in culture and in *in vivo* models of breast cancer growth in order to more effectively intervene with molecularly targeted therapy for breast cancer.

List of Personnel

The personnel involved in completing the work are:

James A. Bennett, PhD
Andres Melendez, PhD
Nicole Lostritto, BS
Ralf-Peter Czekay, PhD
Joseph Mazurkiewicz, PhD

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Supporting Data

Figure Legends:

Fig 1.

HS578T tumor cells were admixed with Hs578Bst fibroblasts so that each cell type was at a concentration of 10^7 cells/ml. 0.2 ml of this admixture was added to 0.1 ml Matrigel and was injected into the mammary fat pad of each female SCID mouse. Injection sites were observed daily, and when tumors were palpable, their size was measured using a Vernier caliper daily for 30 days. AFPep (400 μ g/kg) or Imatinib (80 mg/kg) were injected i.p. once daily beginning one day after tumor implantation. There were five replicate mice per group. * indicates significantly different from control, $p < 0.05$ by Dunnett's Test.

Figs 2 & 3

Cells were grown to 70% confluence in the indicated column, they were transiently infected with c-kit for 24 hr. They were then washed, harvested by scraping off the dish, and then lysed by sonication in hypotonic buffer as previously described (8). Cell free supernatants were obtained after centrifugation of lysates. Protein level was obtained by Bradford assay. Proteins were separated on discontinuous SDS-PAGE 7.5% gels and then transferred by electroblotting to PVDF membranes. After blocking with 5% milk in Tris buffered saline containing 0.1% Tween, the membranes were incubated with primary antibody overnight, washed, secondary antibodies conjugated to HRP were added for 1 hour, washed, and developed using Western lightning for detection by chemiluminescence.

Figs 4 & 5.

Fibroblasts were stained with CellTracker Green BODIPY (Invitrogen), washed, and 5×10^4 fibroblasts were resuspended in 1.8 mg/ml Type 1 collagen, and layered onto Mat Tek dishes. Tumor cells were stained with CellTracker Red CMTPX (Invitrogen), washed, and 2×10^5 cells were added on top of the solidified layer of collagen-containing fibroblasts. Cells were incubated for two days, and images were taken every 24 hours using a Zeiss LSM 510 META-NLO confocal microscope. Experiments were repeated at least three times. Representative images are shown.

Fig 6.

Commercially available transwell chambers containing 8- μ m pore membranes coated with Matrigel were used. The Matrigel-coated membrane separates two, upper and lower, buffer compartments presenting a migration barrier for non-invasive cells. 5×10^4 cells/well were seeded onto the top of the coated filter in serum-free culture medium, and the lower side of the filter was exposed to medium containing 5% serum for 18 h at 37°C. Serum is the stimulus for migration/invasion in this assay. AFPep, Imatinib or vehicle were added at the indicated concentrations to the tumor cells in the upper chamber at the time of seeding. At the end of the incubation, only cells which had invaded through the Matrigel layer to the bottom of the filters were fixed (4% paraformaldehyde), stained (Crystal Violet), and counted over five randomly chosen areas of the filter using a microscope. Crystal Violet was extracted and absorption at 590 nm was also evaluated. Wells were set up in triplicate. Mean cell number and absorption for each group were determined. Differences between groups were similar using either cell

number or absorption. Absorption data were used to calculate % of control. * indicates significantly different from vehicle controls at $p < 0.05$ by Dunnett's Test.

Fig 7.

7×10^4 cells were added to each well of 6-well collagen I-coated plates. Vehicle, AFPep, or Imatinib were added daily at the concentrations indicated. Media were changed every other day immediately before addition of drugs. After 8 days of culture, viable cell numbers were evaluated by MTT assay. Six replicate cultures were established for each condition. Drug treated groups were compared to the vehicle group to calculate percent growth inhibition. When mean cell numbers were compared in each group, inhibitions greater than 25% were significantly different ($p < 0.05$) from control by Dunnett's Test.

Figures:

Fig. 1A In vivo Hs578Bst + Hs578T Growth Data

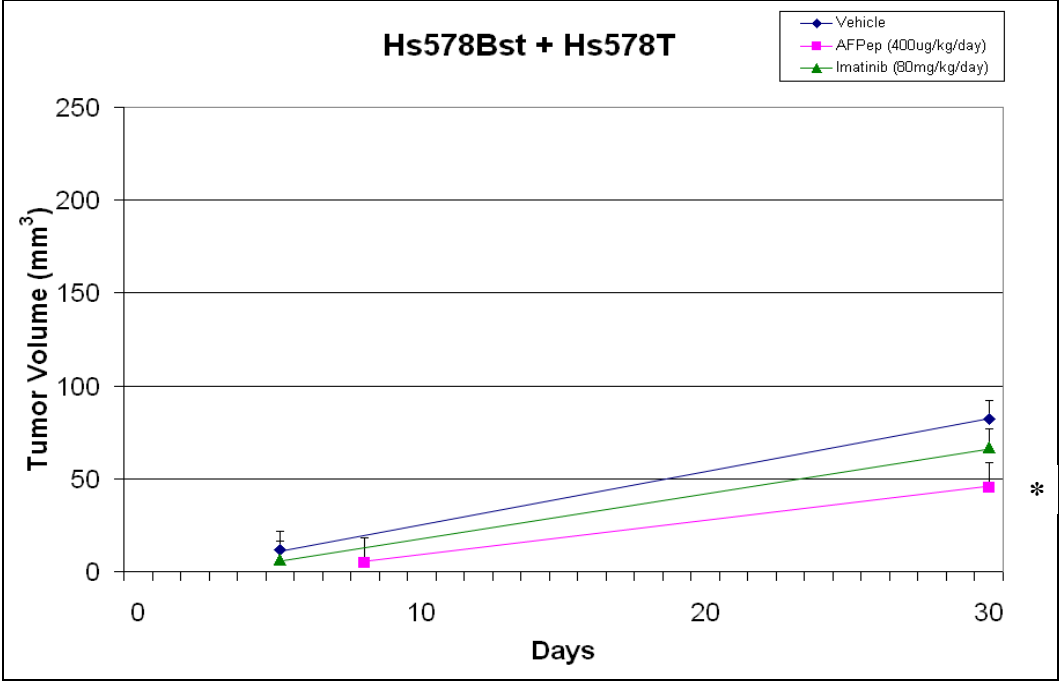


Fig. 1B In vivo c-Kit Hs578Bst + Hs578T Growth Data

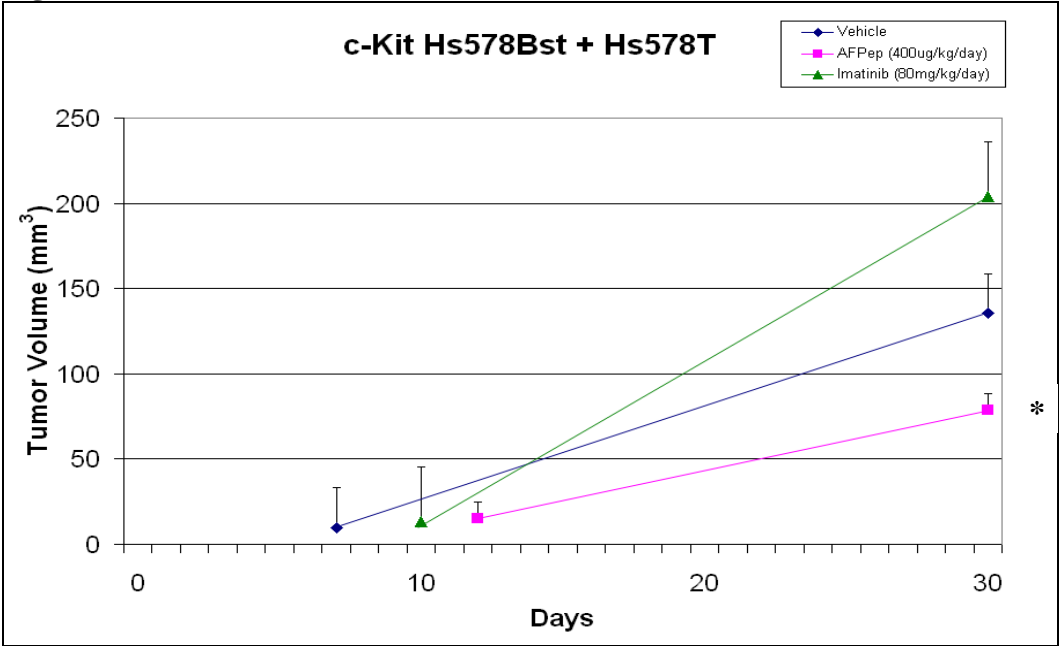


Fig. 2 Western Blot of c-Kit.

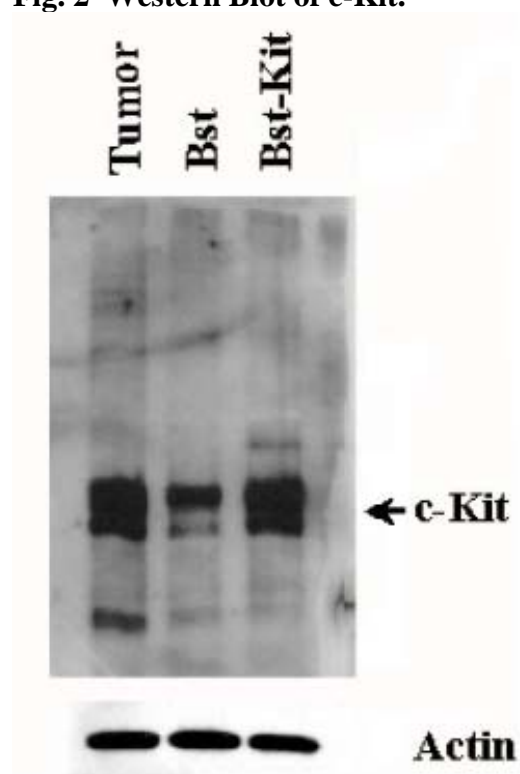


Fig. 3 Western Blot of AKT.

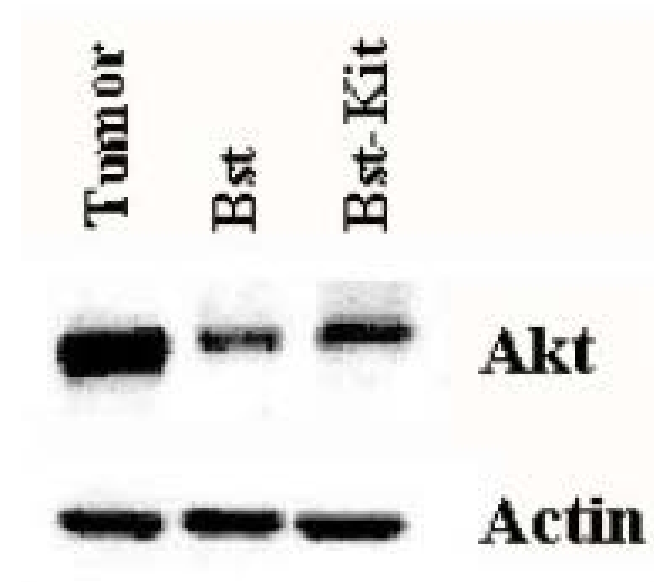


Fig. 4A Hs578Bst Alone, Top View

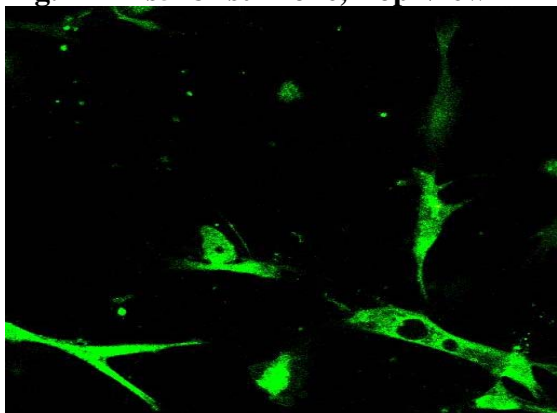


Fig. 4B c-Kit Hs578Bst Alone, Top View

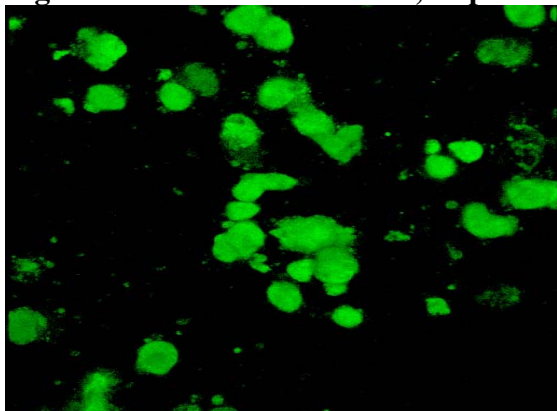


Fig. 4C Hs578T Alone, Top View

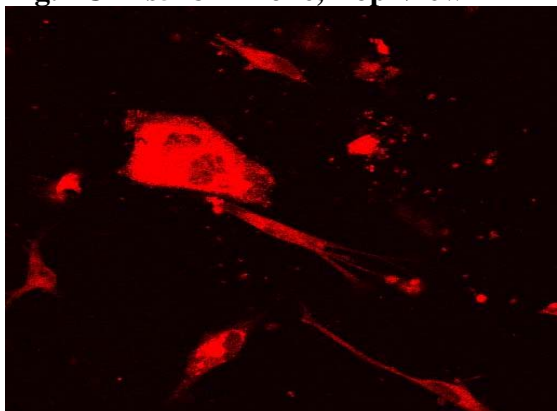
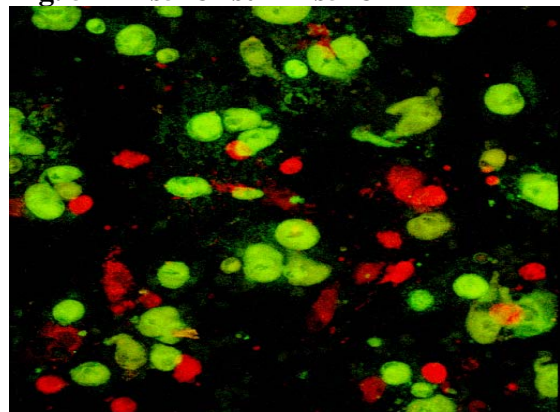
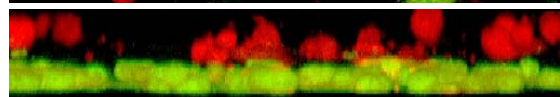


Fig. 5A Hs578Bst + Hs578T

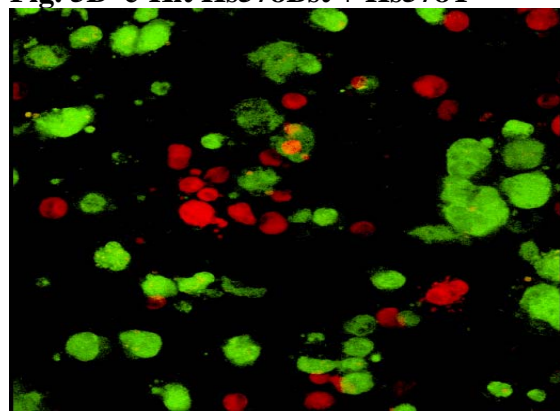


Top View

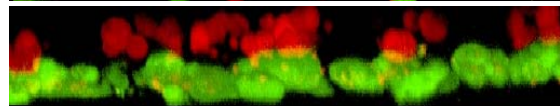


Side View

Fig. 5B c-Kit Hs578Bst + Hs578T

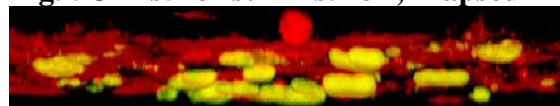


Top View



Side View

Fig. 5C Hs578Bst + Hs578T, Elapsed Time



Side View

Fig. 5D c-Kit Hs578Bst + Hs578T, Elapsed Time

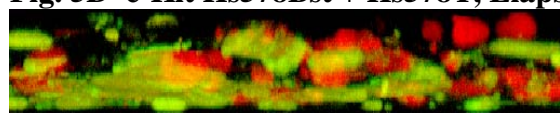
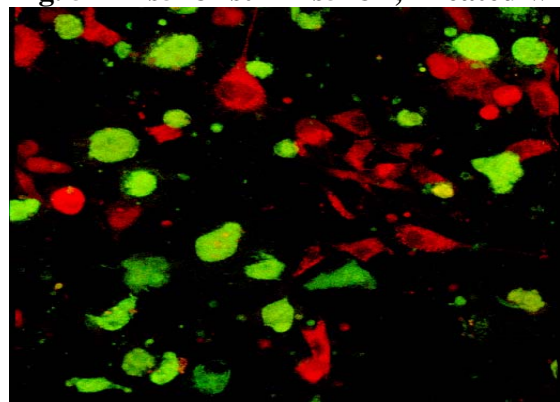
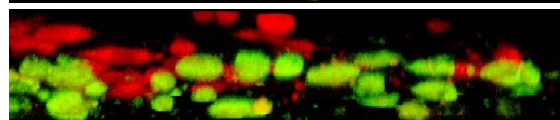


Fig. 5E Hs578Bst + Hs578T, Treated with AFPep

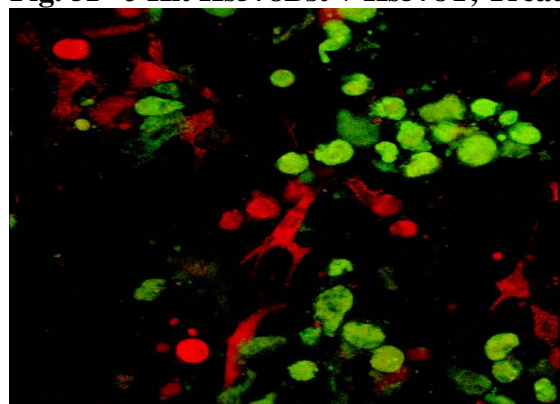


Top View

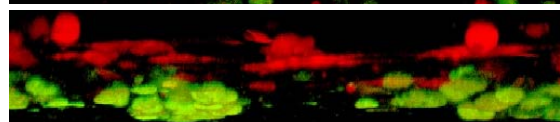


Side View

Fig. 5F c-Kit Hs578Bst + Hs578T, Treated with AFPep

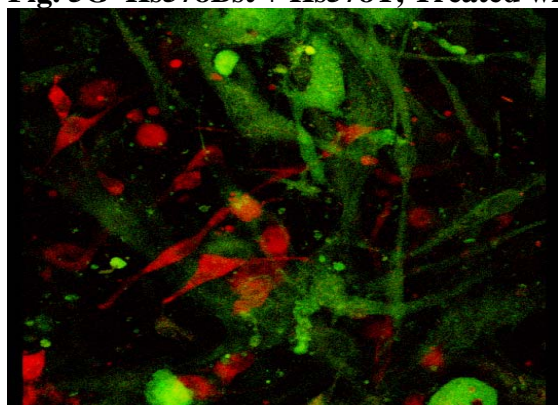


Top View

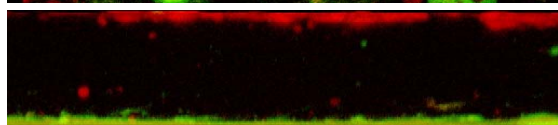


Side View

Fig. 5G Hs578Bst + Hs578T, Treated with Imatinib

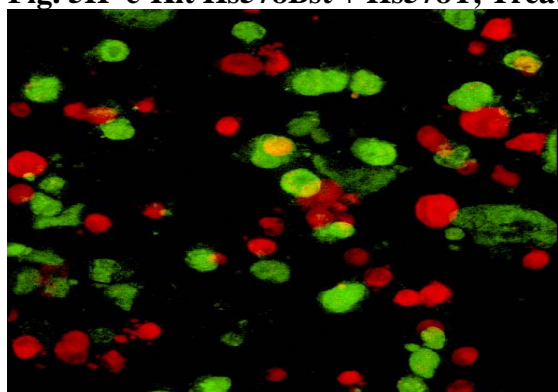


Top View

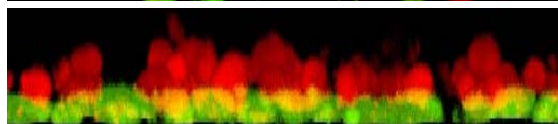


Side View

Fig. 5H c-Kit Hs578Bst + Hs578T, Treated with Imatinib

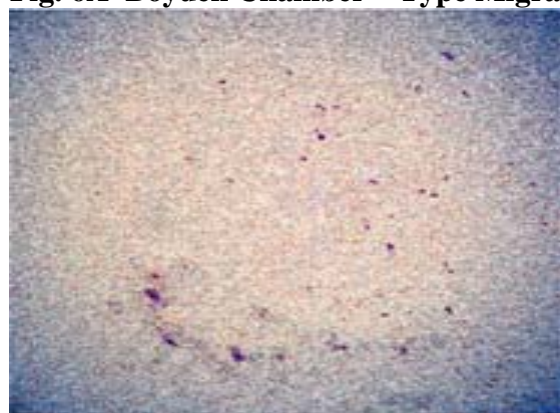


Top View

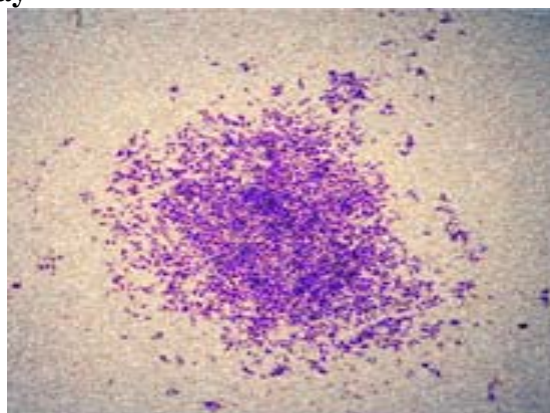


Side View

Fig. 6A Boyden Chamber – Type Migration Assay

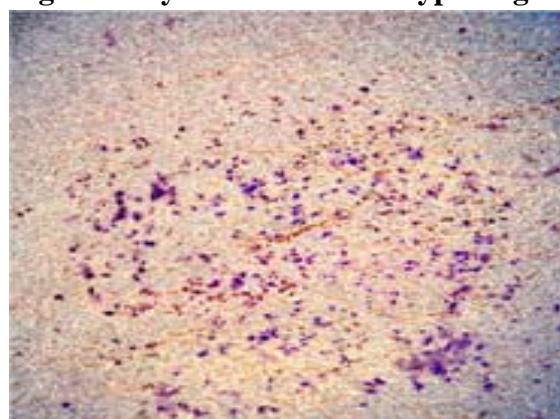


Serum Free

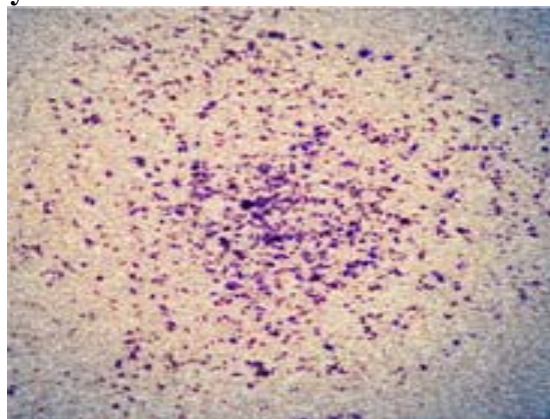


Serum

Fig. 6B Boyden Chamber – Type Migration Assay



Serum + AFPep



Serum + Imatinib

Fig. 6C Inhibition of Hs578T in the Boyden Chamber – Type Migration Assay

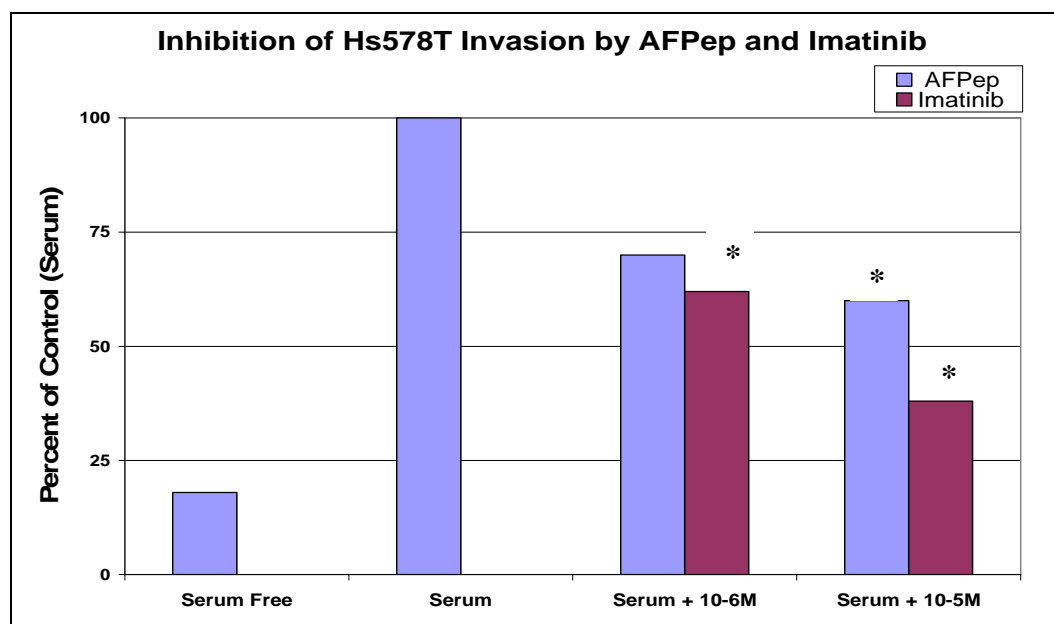


Fig. 7A Hs578Bst Growth Inhibition in Culture

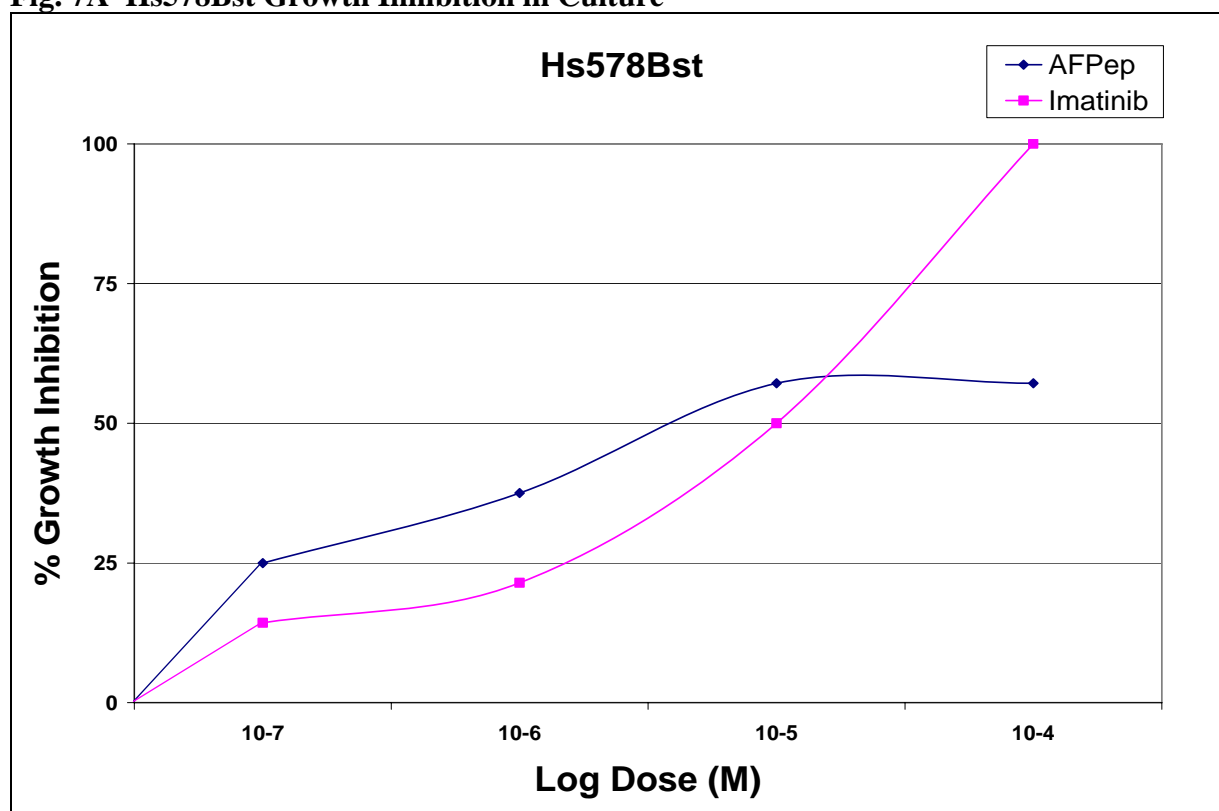


Fig. 7B c-Kit Hs578Bst Growth Inhibition in Culture

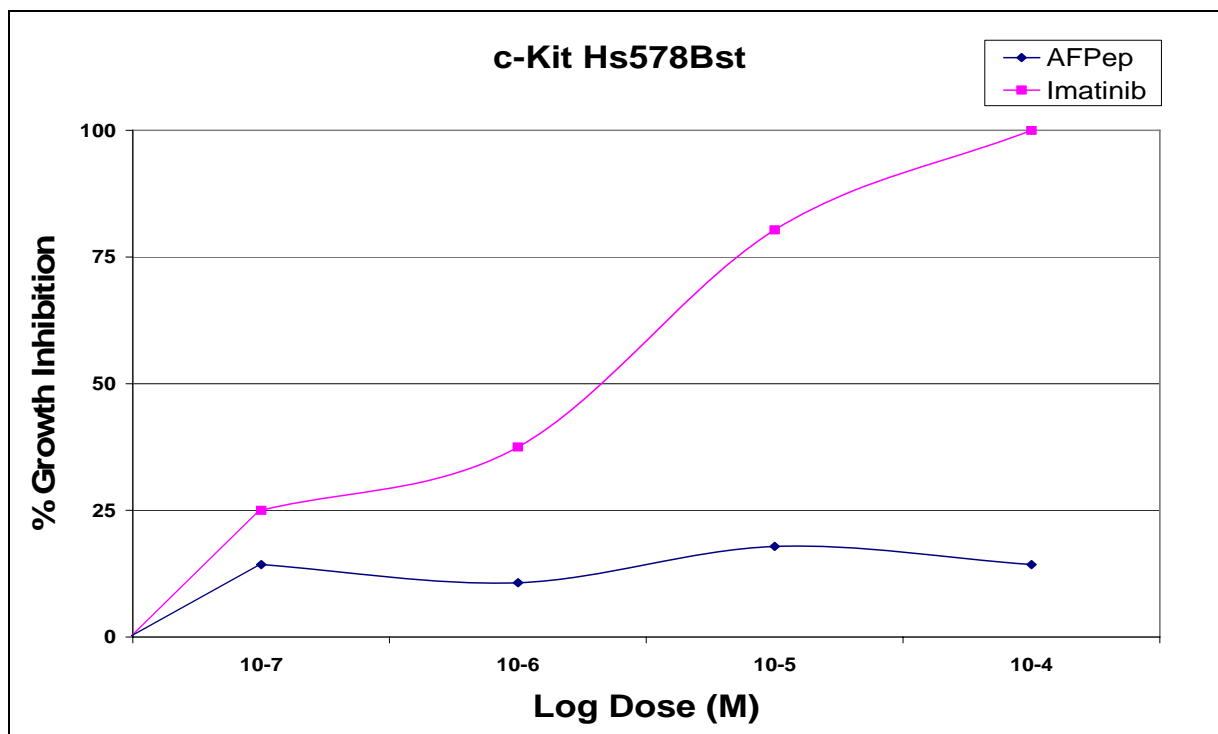


Fig. 7C Hs578T Growth Inhibition in Culture

